IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent application of Hui ZHANG *et al.*	:	Group Art Unit: 1648
Serial No.:	10/688,100	:	Examiner: Jeffrey J. Stucker
Filed:	October 17, 2003	:	
For:	MULTIMERIZATION OF HIV-1 VIF PROTEIN AS A THERAPEUTIC TARGET	:	Confirmation No. 1901

DECLARATION OF ROBERT W. BUCKHEIT, JR. PH.D.
UNDER 37 CFR § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Robert W. Buckheit, Jr. Ph.D. declare as follows:

1. I am President and Chief Scientific Officer of ImQuest BioSciences. The following experimental work was carried out under my supervision, pursuant to a contract between ImQuest BioSciences and OyaGen, Inc.

CERTIFICATE OF MAILING
UNDER 37 C.F.R. 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date indicated below, with sufficient postage, as first class mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

BY Jennifer R. Warren

DATE: 12/7/06

2. The following peptides were tested in this study:

Peptide 1: Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Asp-Leu-Gly-Glu-Gln-His-Phe-Lys-Gly-Leu-Val-Leu

Peptide 2: Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Ser-Asn-Gln-Gly-Gly-Ser-Pro-Leu-Pro-Arg-Ser-Val

3. Peptide 2 comprises the twelve amino acid sequence Ser-Asn-Gln-Gly-Gly-Ser-Pro-Leu-Pro-Arg-Ser-Val, linked to the TAT peptide Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly. Peptide 1 contains the irrelevant control peptide Asp-Leu-Gly-Glu-Gln-His-Phe-Lys-Gly-Leu-Val-Leu, linked to the same TAT peptide.

Materials and Methods

4. In the experiments that follow, Peptide 1 or Peptide 2 was dissolved in water to a final concentration of 20 mM. The stock solutions were diluted to a concentration equivalent to two times the final in-well high test concentration (50 μM) in the appropriate assay medium. The positive control compound AZT was solubilized at a concentration of 1 mM and tested in parallel at a concentration of 1 μM.

5. MT-2 cells and the laboratory-adapted strain HIV-1_{IIIB} were obtained from the NIAID AIDS Research and Reference Reagent Program, Rockville, Maryland. MT-2 cells were infected with HIV-1_{IIIB} according to standard protocols in 96-well microtiter plates. Briefly, 50 μL of compounds and solvent control at high-test concentrations or dilution were added in triplicate to 50 μL of MT-2 cells plated at a density of 2.5×10^3/well. HIV-1_{IIIB} was added to the wells in a volume of 100 μL at four different multiplicities of infection (MOIs) (0.10, 0.032, 0.01 and 0.0032 μL/well). Virus replication in the infected MT-2 cells was monitored daily by reverse transcriptase (RT) assay, described below. Cultures were sub-cultured on day 8 and day 14 by performing a 1:5 split of the culture to maintain the cells in a logarithmic growth mode.

6. Reverse transcriptase was measured in cell-free supernatants using a standard radioactive incorporation polymerization assay. One μL tritiated thymidine triphosphate (NEN;

1 Ci/mL) was used per enzyme reaction. Poly rA and oligo dT were prepared at concentrations of 0.5 mg/mL and 1.7 Units/mL, respectively, from a stock solution which was kept at -20°C. The RT reaction buffer was prepared fresh on a daily basis and consisted of 125 µL of 1 mol/L EGTA, 125 µL of dH_2O, 125 µL of 20% Triton X-100, 50 µL of 1 mol/L Tris (pH 7.4), 50 µL of 1 mol/L DTT, and 40 µL of 1 mol/L $MgCL_2$. For each reaction, 1 µL of TTP, 4 µL of dH_2O, 2.5 µL of rAdT and 2.5 µL of reaction buffer were mixed. Ten microliters of this reaction mixture was placed in a round bottom microtiter plate and 15 µL of virus containing supernatant was added and mixed. The plate was incubated at 37°C in a humidified incubator and incubated for 90 minutes. Following reaction, 10 µL of the reaction volume was spotted onto a DEAE filter mat in an appropriate plate format, washed five times for 5 minutes each in a 5% sodium phosphate buffer, two times for 1 minute each in distilled water, two times for 1 minute each in 70% ethanol, and then air dried. The dried filtermat was placed in a plastic sleeve and 4 mL of Opti-Fluor O was added to each sleeve. Incorporated radioactivity was quantified utilizing a Wallac 1450 Microbeta Trilux liquid scintillation counter.

7. Peptides 1 and 2 were evaluated in parallel at a high test concentration of 50 µM over the course of a 20 day experiment. Water was evaluated in parallel as a control because of the amount of water added as the solvent to the stock peptides. Infection with HIV-1_{IIIB} was initiated a low MOI. The experiments involved evaluation of the test peptides in cells infected with 4 different MOIs: 0.10, 0.03, 0.01 and 0.003.

8. Peptides 1 and 2 and the AZT positive control drug were replenished every other day by adding the fixed concentration back to the well in the volume of supernatant removed from the well. This volume was chosen to assure that fresh peptide was added irrespective of the decay in peptide in the wells but without resulting in the potential for increasing the peptide concentration above 50 µM. Thus, it may be assumed that the fixed concentration of peptide in the wells was actually 25 µM, or ranged from 25-50 µM, depending on the stability of the peptide in the culture.

9. The cells were sub-cultured on day 8 and day 14. The sub-culturing involved the transfer of 20% of the volume of the well (cells and medium) into a well containing fresh tissue culture medium with fresh peptide.

Results and Discussion

10. At high MOI (0.10), little difference was observed between Peptide 1 (control peptide), the virus control and water-treated cultures (**Figure 1).** In each of these samples, virus replication initiated at about day 3 and peaked on day 8. Peptide 2, on the other hand, was clearly inhibitory to virus replication n the cultures, delaying the onset of virus production for approximately 4 to 5 days and peaking at day 11. AZT was completely suppressive for the entire course of the assay.

11. At mid-high MOI (0.03), no protection was observed with Peptide 1 treatment of the infected cells (**Figure 2).** It appeared that virus replication may have been slightly enhanced with regard to the time to onset of virus replication in the culture compared to the virus control. In the Peptide 2 treated cultures, a 2 to 3 day delay to the onset of virus production was observed. AZT was completely suppressive at all time points tested.

12. At mid-low MOI (0.01) a comparison of Peptide 1 and Peptide 2 clearly demonstrated the suppressive effect of Peptide 2, delaying the onset of virus production by 3 to 4 days and reducing the peak levels of virus produced in the culture by 50% **(Figure 3).** Peptide 1, water and the virus control curves appear identical, suggesting that Peptide 1 and water did not actively inhibit virus production in these cultures. AZT was completely suppressive at all time points evaluated.

13. At the lowest MOI evaluated (0.003), Peptide 1 induced virus production in the cultures, compared to Peptide 2 which appeared to mimic the virus control curves **(Figure 4).** AZT was completely suppressive at all time points tested.

14. The results indicate that Peptide 2 is suppressive at least at the three highest MOIs evaluated, whereas the control peptide, Peptide 1, is not suppressive at any of the MOIs evaluated.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11/30/06
(date)

ROBERT W. BUCKHEIT, JR. PH.D.

Figure 1: Efficacy of Peptide 1 and Peptide 2 at High MOI Infection (0.1)

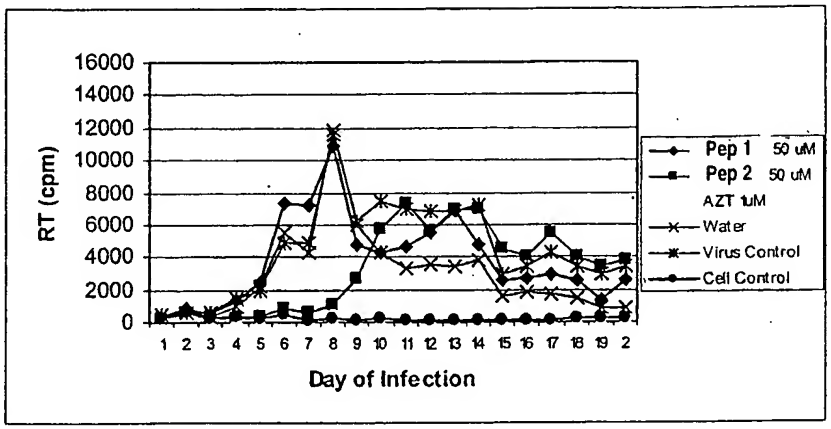

Figure 2: Efficacy of Peptide 1 and Peptide 2 at Mid-High MOI (0.03)

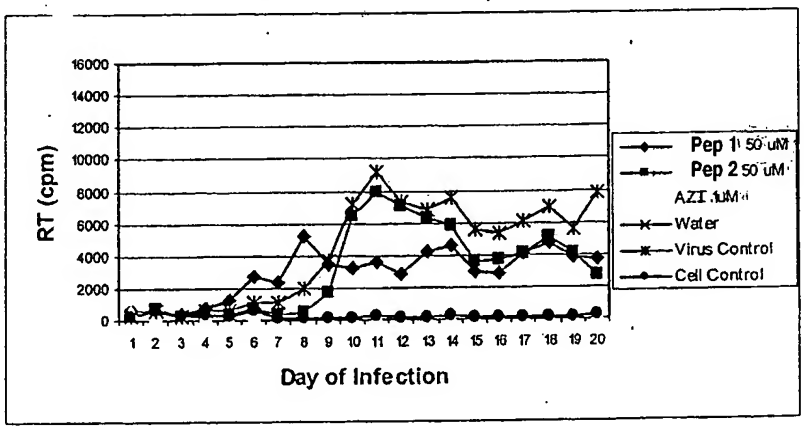

Figure 3: Efficacy of Peptide 1 and Peptide 2 at Mid-Low MOI (0.01)

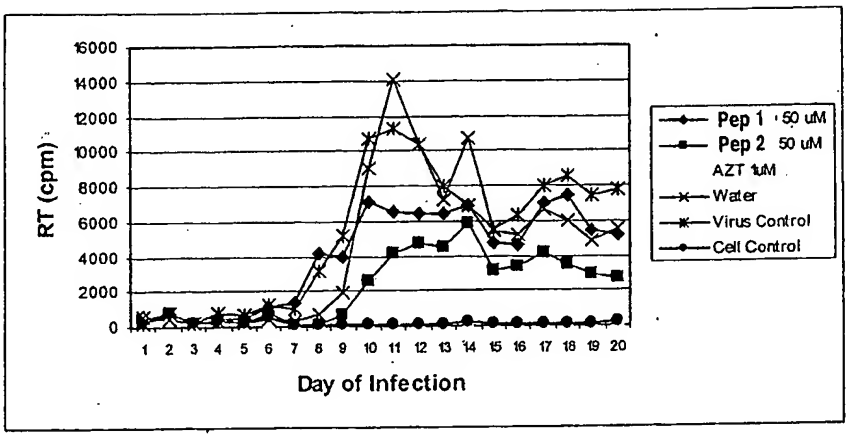

Figure 4: Efficacy of Peptide 1 and Peptide 2 at Low MOI (0.003)